3. Results
3.1. Tetracycline-inducible expression of EhCaBP1 in HM1: IMSS

3.1.1. Vector Description

The tetracycline-inducible expression vector pEhHYG-tetR-0-CAT was used to transcribe the EhCaBP1 gene in both the sense and the antisense orientations. This expression vector is schematically shown in Fig. 6A. This vector uses tetracycline-regulatable operon of *E. coli* (Hamann *et al.*, 1997) for inducible expression of a foreign gene in *E. histolytica*. In this system a tetracycline-responsive tet-repressor binds to the tet-operator resulting in repression of those genes that are driven by promoters bearing this operator sequence. The genes can be switched on in presence of tetracycline at non-toxic concentration as it abolishes binding of the repressor to the operator. Since the repressor gene is flanked by amebic 5' lectin promoter sequence and by downstream actin 3' sequences it is constitutively expressed in amebic cells. The vector has a hygromycin resistance gene for selection, which is flanked by amebic actin promoter and downstream sequences. The prokaryotic chloramphenicol acetyltransferase (CAT) gene is flanked by KpnI and BamHI sites which can be replaced by the gene of interest. It is also under control of lectin promoter and downstream actin sequences of *E. histolytica*.

3.1.2. PCR amplification and cloning in pEhHYG-TetR-O-CAT vector

PCR primers were designed to incorporate KpnI and BamHI sites in EhCaBP1 gene for cloning in pEhHYG-TetR-O-CAT vector. For sense orientation KpnI and BamHI sites were incorporated at 5' and 3' end respectively, and vice versa for cloning the gene in opposite orientation. The CAT gene was excised from pEhHYG-TetR-O-CAT vector with KpnI and BamHI and then replaced with EhCaBP1 gene in either orientation as shown in Fig. 6B. The orientation of the insert in the recombinant plasmids, pEhCaBP1-S and pEhCaBP1-AS, was confirmed by restriction mapping. HindIII site is located at the 5' end of EhCaBP1 giving rise to different sized bands depending on the orientation. The sizes of bands obtained were 7.4 kb and 0.95 kb from pEhCaBP1-S and 7.74 kb and 0.663 kb from pEhCaBP1-AS respectively (Fig. 7B). The sizes matched with expected values (Fig. 7A) and the orientations of inserts were further confirmed by nucleotide sequencing.
Fig. 5

A. Schematic diagram of pHYG-TetR-O-CAT. The upstream and downstream flanking regions for hygromycin resistance cassette, tetracycline repressor protein and reporter gene CAT are indicated.

B. The strategy for cloning EhCaBP1 in different orientations. CAT gene is replaced by EhCaBP1 gene at KpnI (k) / BamHI (b) site in sense and reverse orientation. The resulting plasmids were named pHYG-CaBP1-S and pHYG-CaBP1-AS respectively.
Expected band sizes in kb: 7.4 and 0.95 for pEhCaBP1-S

7.74 and 0.663 for pEhCaBP1-AS

8.7 kb for pEhHYG-TetR-O-CAT

Fig. 7. Analysis of pEhCaBP1-S and pEhCaBP1-AS by restriction enzyme digestion.  
A. Schematic diagrams of the plasmids and expected digestion pattern with HindIII.  
B. Plasmids were digested with HindIII and analyzed by agarose gel electrophoresis. 1% agarose gel (w/v) at 3 V/cm was used for separation. Lanes are: 1, 1 kb ladder; 2, pEhHYG-TetR-O-CAT; 3, pEhCaBP1-S; 4, pEhCaBP1-AS; 5, Marker. Marker band sizes are indicated to the left and right side of the gel.
3.1.3. Transfection of *E. histolytica* trophozoites and selection by hygromycin resistance

Initially parental plasmid vector pEhHYG-TetR-O-CAT was used for transfection. Since hygromycin is used for positive selection with this vector, it was important to demonstrate that the HM-1:IMSS cells maintained by us were sensitive to this antibiotic. When *E. histolytica* cells were incubated with hygromycin at 5 μg/ml, no live cells were found after 48 hours indicating that these are sensitive to hygromycin. For transfection, normally 200 μg of circular plasmid DNA was mixed with \(10^7\) cells and the plasmids were introduced by electroporation. The cells carrying the plasmids were selected by growing in presence of hygromycin at 10 μg/ml. In general a transformed cell line was obtained after two weeks of electroporation. When transfection was carried out in absence of the plasmid no live resistant amoeba was obtained suggesting that the transformed phenotype is not because of some changes due to electroporation. Presence of plasmid DNA in transformed cells was checked by Southern hybridization using bacterial component of the plasmid as probe, such as the vector backbone fragment of pEhHYG-TetR-O-CAT.

*E. histolytica* cell lines carrying the two chimeric plasmids pEhCaBP1-S and pEhCaBP1-AS were named EhCaBP1-S and EhCaBP1-AS respectively. The transformed cell-lines were maintained in presence of hygromycin at 10 μg/ml. Southern blots of the total genomic DNA prepared from transformed cells were hybridized with \(^{32}\text{P}\)-labeled vector backbone fragment (EcoRI and XbaI released) as shown fig. 8A. The autoradiogram showed two bands of \(\sim 8\) and 12 kb respectively consistent with the suggestion that the plasmid DNA was not integrated to the ameba genome but remained episomally as unrearranged circular plasmid in relaxed and concatameric forms (Fig. 8B, lane 1 and 2). This was further confirmed by restriction enzyme analysis. When the same probe was used to hybridize EcoRI digested genomic DNA, a single band of 3.8 kb was observed in EhCaBP1-S cells. Correspondingly a 4.2 kb band was observed for EhCaBP1-AS cells (Fig. 8B). The pattern matched with the expected values obtained if the plasmids are maintained as episomal entities (Fig. 8A).

The copy number of the two plasmids increased with increasing concentration of hygromycin (10, 20, 40 μg/ml) used for maintaining transformed cells. This was checked by hybridization with the \(^{32}\text{P}\)-labeled vector backbone probe, using the same amount of genomic DNA (Fig. 59).
Fig. 8. Southern hybridizations of transfected HM-1:IMSS trophozoites.
A. Schematic depiction of plasmids pEhCaBP1-AS and pEhCaBP1-S and the expected fragment sizes generated by digestion with EcoRI.
B. EhCaBP1-S or EhCaBP1-AS cells were grown in presence of 10 μg/ml hygromycin. The genomic DNA was isolated and then separated on a 0.8% agarose gel at 3V/cm for 4 h. The Southern blot was hybridized with the ^32P-labeled EcoRI and Xbal released vector backbone fragment as shown in figure. Lanes are: 1, EhCaBP1-AS; 2, EhCaBP1-S; 3, EhCaBP1-AS; 4, HM1:IMSS and 5, EhCaBP1-S.
C. EhCaBP1-S and AS cells were grown in presence of indicated concentrations of hygromycin for 60 h. The genomic DNA was isolated and was used to prepare dot blots containing 200 ng of DNA. The blot was hybridized with the above mentioned probe.
Results

8C). Autoradiogram showed an increase in hybridization signal with increasing concentration of hygromycin, nearly saturating at 40 μg/ml. When 50 μg/ml hygromycin was used the cells were found to grow very slowly. Since there was not much difference in plasmid content (about 2X) between 10 and 40 μg/ml, most of the subsequent experiments were carried out at 20 μg/ml hygromycin as the cells were much healthier compared to that observed in presence of higher concentration of the drug.

3.1.4. Expression of EhCaBP1 in transfected trophozoites

RNA level

The expression of EhCaBP1 in the transformed lines was checked by northern hybridization using either double stranded- or strand specific-probes. Since EhCaBP1 gene is under the control of tetracycline therefore on addition of tetracycline it is expected that there will be an increase in transcript level. The northern blots were hybridized with the EhCaBP1 probe (double stranded) and the results showed about four fold increase in the EhCaBP1 mRNA levels in EhCaBP1-S cells after addition of tetracycline (Fig. 9A). In presence of tetracycline there was a two fold decrease in the expression level of EhCaBP1 in EhCaBP1-AS cells (Fig. 9B). As expected, no detectable RNA was observed in EhCaBP1-S cells in presence or absence of tetracycline when antisense-strand-specific probe was used for hybridization (Fig. 9C). An intense band of antisense RNA was observed in EhCaBP1-AS cells on induction by tetracycline. There was no detectable signal in absence of tetracycline. This suggests that the tetracycline regulatable system in E. histolytica may have a tight expression control. Expression of actin genes was found to be similar in all the samples suggesting that the changes observed in EhCaBP1 expression were specific (Fig. 9D). The possibility that the antisense-mediated inhibition in the expression was due to RNAi-based mechanism was ruled out as no short specific RNA product derived from EhCaBP1 was observed when total RNA from uninduced and tetracycline induced EhCaBP1-AS trophozoites were separated on a 15% urea-acrylamide gel and hybridized with EhCaBP1 (data not shown).

Protein level

Expression of EhCaBP was also measured at the protein level by both Western blot (Fig. 10A.1) and immunoprecipitation (Fig. 10D) techniques. For immunoprecipitation the cells were first
Fig. 9. Expression of EhCaBP1 in transfected cells by Northern analysis. RNA was isolated from indicated cells grown in presence of 20 μg/ml hygromycin with or without 5 μg/ml tetracycline (added at 0 h) for 48 h. For each analysis 20 μg of total RNA was used and separated on a 1% agarose gel at 3V/cm for 3 h by glyoxal/DMSO method as described in Materials and Methods. Hybridization was carried out with [32P] labeled EhCaBP1 or actin gene probes. Random and strand specific probe labelings were done following the protocol described in Materials and Methods. The blots were exposed for different time period depending on the required level of intensity. Probe types used are: double stranded EhCaBP1 fragment (A), EhCaBP1 sense strand specific fragment (B), EhCaBP1 antisense strand specific (C) and with actin probe (D). Detailed protocol for strand specific labeling is described in Materials and Methods. The EhCaBP1 probe recognizes a 0.41 kb band and actin a 1.1 kb band.
A.1
Tetracycline: - + - +
EhCaBP1-AS EhCaBP1-S

A.2
Tetracycline: - + - +
EhCaBP1-AS EhCaBP1-S

C

Mean Pixel Value

6 h 12 h 24 h 48 h 60 h

Time (in hours)

B

D

Tetracycline: - + - +
EhCaBP1-AS EhCaBP1-S

Fig. 10.
A.1. Expression of EhCaBP in transfected cells. Transfected cells were grown in presence of 20μg/ml hygromycin and 5 μg/ml tetracycline for 48 h. Equal amount (200 μg) of cellular lysate from indicated cells were separated on a 12% SDS-PAGE gel and were electrophoretically transferred onto a nitrocellulose membrane as described in Materials and Methods. EhCaBP was identified by immunostaining with rabbit anti-EhCaBP antibody at 1:1000 dilution. The second antibody used was goat anti-rabbit AP conjugate and staining was done with BCIP/NBT.
A.2. Coomassie blue staining of SDS-PAGE gel, run under above said conditions.
B. Histogram showing protein expression level in pixels of the bands from three independent western analysis.
C. Time course of EhCaBP protein expression on addition of tetracycline to EhCaBP1-S cells. In this 3X10⁶ cells were used for each time point. Mean pixel value of the EhCaBP band recognized by anti-EhCaBP antibody from western analysis was calculated and plotted for the histogram.
D. Immunoprecipitation of EhCaBP. Transfected cells grown for 48 h were radiolabeled with [35S] methionine and the cellular lysate was incubated with anti-EhCaBP antibody as described in Materials and Methods. The immune complex was separated by Protein A agarose beads and analyzed by SDS-PAGE (12% acrylamide) gel followed by fluorography.
Results

labeled with [35S]methionine before preparation of lysates. All comparisons were made against cells grown without tetracycline. Both western blot and immunoprecipitation analysis showed substantial decrease in EhCaBP levels (2 and 2.7 fold respectively) in EhCaBP1-AS cells in presence of tetracycline. On addition of tetracycline there was a steady increase in the expression of EhCaBP in EhCaBP1-S cells reaching saturation by 48 hours (Fig. 10C). At saturation there was a 4-fold increase in EhCaBP levels in EhCaBP1-S cells (Fig 10B). Since the antibody used here recognized both the isoforms of EhCaBP it was difficult for us to indicate the level of expression of only EhCaBP1.

The data suggest that the expression of antisense RNA lead to decreased levels of expression of both EhCaBP1 mRNA and protein. The level of inhibition was found to be between 50-80% in different experiments. The observed inhibition of protein synthesis is not due to of the effect of tetracycline directly on protein synthetic apparatus as no significant inhibition was obtained in total [35S]methionine incorporation after 24 h of tetracycline induction (Fig. 13A). These cells also did not show any significant change in the expression of an unrelated molecule, such as the proteophosphoglycans (data not shown).

3.1.5. EhCaBP1 and EhCaBP2 levels in transformed cells.

It was possible to monitor expression of a specific isotype of EhCaBP by using high stringency hybridization in northern analysis. While the expression of EhCaBP1 was inhibited in EhCaBP1-AS cells in presence of tetracycline there was no change in levels of EhCaBP2 mRNA. No detectable level of EhCaBP2 antisense RNA was observed in these cells. This suggests that the effect on EhCaBP1 expression is specific inspite of high level of similarity between the two isoforms (Fig 11A).

In order to study the expression at the protein level antibody against EhCaBP1 was made specific by absorption against EhCaBP2-Sepharose. The specificity of the antibody is shown in Fig 11B. The absorbed antibody recognized only EhCaBP1 in western blots. This antibody was used to analyze EhCaBP1-S cells growing in presence of tetracycline using western blots (Fig 11C). The overexpressed level of EhCaBP1 in EhCaBP1-S cells on tetracycline induction was seen, though the level without tet-induction or in antisense-blocked cells failed to show up with
Fig. 11. Expression of EhCaBP1 and EhCaBP2.
A. Northern analysis. RNA was isolated from pEhCaBP1-AS cells grown in presence of 20 μg/ml hygromycin with or without 5 μg/ml tetracycline (added at 0 h) for 48 h. For each analysis 20 μg of total RNA was used and separated on a 1% agarose gel at 3V/cm for 3 h by glyoxal/DMSO method as described in Materials and Methods. Hybridization was carried out with [32P] labeled EhCaBP1 or EhCaBP2 coding region. Probe types used are: sense strand specific fragment and antisense strand specific fragment of EhCaBP1 and EhCaBP2 and actin probe. Probe preparation is described in Materials and Methods. The EhCaBP1 and EhCaBP2 probe recognizes a 0.41 kb band and actin a 1.1 kb band.

B. Western Analysis. EhCaBP1 was identified by immunostaining with rabbit anti-EhCaBP1 specific antibody purified by passing through EhCaBP2-Sepharose column, used at 1:1000 dilution. Equal amount (2 μg) of EhCaBP1 and EhCaBP2 were separated on a 12% SDS-PAGE gel and were electrophoretically transferred onto a nitrocellulose membrane as described in Materials and Methods. The second antibody used was Peroxidase linked anti-rabbit antibody and staining was done with BCIP/NBT. Right panel shows Coomassie blue staining of SDS-PAGE gel, run under above said conditions.

C. Expression of EhCaBP1 in EhCaBP1-S cells. Cells were grown in presence of 20 μg/ml hygromycin and with or without 5 μg/ml tetracycline for 48 h. Equal amount (200 μg) of cellular lysate were separated on a 12% SDS-PAGE gel and transferred onto a nitrocellulose membrane for western analysis with anti-EhCaBP1 specific antibody as above.
EhCaBP1 specific antibody. This suggests that EhCaBP1 is present in very low quantity in *E. histolytica* hence detection is possible only in over-expressing cells.

3.2. Cell proliferation and macromolecular synthesis

It is likely that altered expression of EhCaBP1 may cause physiological changes in the transformed cell lines. Cellular proliferation is a key indicator for physiological changes in cells. In order to identify phenotypic changes in these cells the changes in cellular proliferation in presence and absence of tetracycline was observed. EhCaBP1-AS Cells grew poorly in presence of tetracycline. Although these cells attained saturation at 72 hours (as in normal cells), cell number was reduced by 50-70% at saturation (Fig. 12A). Similar results were obtained in two independently transformed cell lines suggesting that this is not due to nonspecific changes introduced at the time of transfection. Cell proliferation was also inhibited when tetracycline was added at mid-log phase (Figure 12C). However, these slow growing cells were viable as these did not take up trypan blue. A slight reduction in cell number (15-30%) was also observed in EhCaBP1-S cells in the presence of tetracycline. However, this was not statistically significant (P value > 0.05). Cells containing the parental plasmid pEhHYG-TetR-O-CAT or without any plasmid showed similar growth pattern in absence and presence of tetracycline (Fig. 12B). Continuous sub-culturing in presence of tetracycline affected growth of cells carrying the pEhCaBP1-S construct. The effect of antisense EhCaBP1 expression on growth was reversible, as removal of tetracycline from the culture restored normal growth.

To test if the effect on cellular proliferation was due to inhibition of macromolecular synthesis, the levels of protein and DNA synthesis were determined by incorporation of [35S]methionine and [3H] thymidine, respectively (Fig. 13A, B). No significant change in the level of protein and DNA synthesis was observed in the expression blocked cells. This suggests that the growth inhibition may not be due to a generalized inhibition of macromolecular synthesis.
Fig. 12. Growth of transfected cells. All cells were grown in presence of 20 µg/ml hygromycin and tetracycline was added to the medium as indicated at 5 µg/ml at 0 h. Cells were grown in 5 ml culture tubes in triplicate for all the experiments and counting was carried out using a haemocytometer, after chilling the tube for 5 min.

A. Transfected trophozoites grown with and without tetracycline.
B. Histogram showing mean cell number after 48 h of growth for different transfected cell-types with and without tetracycline. Vector refers to pEHHYG-TetR-O-CAT
C. Growth curve of EhCaBP1-AS cells where tetracycline was added at 36th h after inoculation.
Fig. 13.
A. Protein synthesis in transfected trophozoites. Transformed cells (10^6) grown in 20 µg/ml hygromycin and were incubated with or without tetracycline for 48 h, were radiolabeled with [35S]methionine for 1 h. Cell lysate was prepared as described in Materials and Methods. Relative cpm is plotted in the histogram.
B. DNA synthesis in transfected trophozoites. [3H]thymidine labeled cell lysate was prepared as above. Relative cpm is plotted in the histogram.
3.3. Phagocytosis

Endocytosis constitutes an important physiological property of *E. histolytica* and is thought to be associated with pathogenesis (Orozco E *et al.*, 1983). Presence of acidic lysosomal compartments by accumulation of acridine orange suggests existence of active endocytic pathway in amoebae (Aley S B *et al.*, 1984). Amoebae pinocytose as much as 30% of their volume per hour and this is the major source of food and nutrients. A defect in the endocytic pathway would affect the growth of these organisms. Erythrophagocytosis has been used as a marker for pathogenic potential of *E. histolytica* cells.

3.3.1 Fluid-phase endocytosis

The involvement of EhCaBP1 in fluid-phase endocytosis was inferred by determining the level of uptake of the fluorescent marker FITC-Dextran in EhCaBP1-AS and EhCaBP1-S cells. A typical cell of each cell-type in absence and presence of tetracycline is shown in figure 14A. The number of vesicles that have taken up the fluorescent molecules were counted. The cells expressing antisense EhCaBP1 showed a substantial decrease in uptake (70%) in presence of tetracycline compared to un-induced cells (Fig. 14B). There was no significant difference in the uptake of FITC-Dextran in EhCaBP1-S cells in presence or absence of tetracycline. This suggests that pinocytosis is inhibited in the antisense-blocked cell line and there may be an involvement of EhCaBP1 in pinocytosis.

3.3.2 Erythrophagocytosis

A spectrophotometric technique was used to measure the quantity of haemoglobin contained in the amoebae that had engulfed RBC. The amount of haemoglobin was a direct measure of erythrophagocytosis. The erythrophagocytosis activity of EhCaBP1-AS cells in absence of tetracycline and EhCaBP1-S cells in presence or absence of tetracycline were found to be comparable. However, erythrophagocytosis decreased by 60% in EhCaBP1-AS cells when grown in presence of tetracycline. The decline in erythrophagocytosis activity was visible within ten min of addition of RBC (Fig. 15A). Number of RBCs taken up can be quantitated by optical density at 400 nm (an optical density of 0.7 at 400 nm corresponds to $3 \times 10^6$ RBC/ml) from a standard curve obtained previously from known numbers of RBCs. EhCaBP1-AS cells engulfed
Fig. 14. Fluid-phase endocytosis in EhCaBP1-AS and EhCaBP1-S cells. Transformed cells were maintained in 20 µg/ml hygromycin and were incubated with or without 5 µg/ml tetracycline for 48 h. The cells were incubated with 1 mg/ml FITC-Dextran in PBS for 30 min followed by thorough washing and mounting on glass slides for viewing under a fluorescent microscope.

A. Fluorescent image of typical cells at 63X magnification.

B. Quantitative analysis of the fluorescent images. Number of vesicles containing fluorescent FITC-Dextran was counted in each cell. The data represent percent value in the mean number of fluorescent labeled vesicles per cell. The cells were randomly selected per slide and counting of engulfed beads was done for five separate slides.
Fig. 15. Erythrophagocytosis in transfected cells. Cells were maintained in hygromycin at 20 μg/ml hygromycin and were treated with tetracycline at 5 μg/ml for 48 h. The cells (10⁶) were incubated with red blood cells (10⁶) for indicated times, and washed with water to remove adhering cells. The red blood cells were then lysed with formic acid and the heme concentration was determined by spectroscopy at 400 nm.

A. The amount of heme as determined by optical density at 400 nm +/- s.d for different cell types; as indicated. EhCaBP1-S, EhCaBP1-S+tet, EhCaBP1-AS and EhCaBP1-AS+tet.

B. The number of RBC internalized, calculated from comparing the measured optical density by a standard curve, generated with known number of RBCs.
Results

about ~ 5 amoebae in 10 minutes in presence of tetracycline in comparison to ~ 10-15 amoebae for other cells (Fig. 15B). The results suggest that both fluid-phase pinocytosis and phagocytosis are inhibited in cells containing reduced amount of EhCaBP1 and therefore this molecule may be involved in these processes.

3.4. Movement trajectory

*E. histolytica* displays typical ameboid movement involving extension and retraction of pseudopods. The motility of transformed cells was determined by optical tracking of a few randomly selected cells in a field, video recording and analysis using a custom-designed software as described in Materials and Methods. Whereas wild type amoebae moved with a mean velocity of 1.4 +/- 0.2 μm/s, the corresponding value for EhCaBP1-AS cells in presence of tetracycline was 0.9 +/- 0.2 μm/s. The mean displacement was also inhibited by about 50% in antisense blocked cells (0.11 μM). A majority of antisense blocked cells wobbled around stationary points of contact with the substratum and seemed to extend short and stumpy pseudopods. Tetracycline induced EhCaBP1-S migrated with velocity of 1.7 +/- 0.4 μm/s and mean displacement of 0.368 μm per second. These cells threw rapid and elongated pseudopods making straight or directional trajectories (Fig. 16).

3.5. Identification of differentially expressed genes by mRNA display

The growth inhibition observed in EhCaBP1-AS cells may be due to reduced expression of specific gene/s. Inhibition in cellular proliferation may be due to inhibition of specific gene/s. In order to identify these target gene/s mRNA display technique was used as described in Materials and Methods and summarized in Fig. 17A. These genes are likely to be downstream targets in the EhCaBP1-mediated signaling pathway.

3.5.1. Reverse transcription, amplification and initial screening

The reverse transcription was carried out using four different anchored-oligo-dTs as described in Materials and Methods. For second round amplification three different decamers were used. On an average about hundred bands were observed per primer pair in each lane after an exposure time of 24 h (Fig. 17B). Overall about 1000 genes spread over 3000 bands were visualized.
Fig. 16. Movement of transfected cells. Images of amoebae were recorded for 5 minutes with a field delay of one second and computer analyzed for their trajectories using conditions described in Materials and Methods. Micrograph of trajectories of few amoebae are shown.
A. EhCaBP1-S cells were grown in presence of tetracycline at 5 μg/ml for 48 h. The inset shows cells with more than 3 pseudopods. Arrows are pointing to pseudopodia.
B. EhCaBP1-AS cells were grown in presence of tetracycline at 5 μg/ml for 48 h. The inset shows cells with one or two pseudopods. Arrows are pointing to pseudopodia.
A
Detection of genes that are differentially expressed in EhCaBP1-AS cells

Amplify mRNAs using anchored oligo dT primers (T12MGA/ATC)

PCR amplification using arbitrary decamers

Separation on PAGE

Select bands with differential intensity, excise, reamplify and confirmation by reverse northern

Molecular cloning in pGEM Teasy vector

Nucleotide sequencing

Confirmation by Northern

---

Fig. 17. Identification of differentially regulated genes by mRNA differential display. EhCaBP1-AS cells were grown for 48 h in presence of 20 μg/ml hygromycin with or without 5 μg/ml tetracycline. Total RNA (5μg) was used for each amplification as described in Methods.

A. Schematic diagram of differential display.
B. A typical PAGE analysis showing displayed amplified cDNA on a 6% DNA sequencing gel.
C. Selected amplicons showing differential levels of mRNA synthesis.
Results

Figure 17C shows some of the differentially displayed bands that were excised for further analysis.

3.5.2. Reverse northern analysis
Thirty five bands were selected from differential displayed profiles, excised and further tested for differential expression using reverse northern approach. In this approach amplified DNA corresponding to different bands were spotted on a nylon membrane and the membranes were hybridized by radiolabeled cDNA probes made from total cellular RNA from EhCaBP1-AS cells growing with or without tetracycline. Some of the reamplified bands are shown in Figure 18A. A typical hybridization is shown in Fig. 18B. Eventually ten bands were selected which showed differential hybridization. These ten promising cDNAs were cloned in pGEM-Teasy vector (Promega) and the nucleotide sequence of each was determined.

3.5.3. Sequence analysis
The identity of the differentially expressed genes was inferred from database searches using the algorithm BLAST (blastn for corresponding GSS entry followed by blastx). Profile and motif searches were also carried out using the Expasy Molecular Biology Server. The results are shown in Table 2. The function of only five of the genes could be predicted. One of the gene was predicted to be a phosphatase based on motif analysis. The sequences are shown in the Appendix 1.

3.5.4. Northern analysis
The differential expression of the three identified genes, namely profilin, ribosomal protein L29 and ribosomal protein S26E were also confirmed by northern blot analysis (Fig 18C). Densitometric scanning of the hybridization signals in the northern blots showed 3-4 fold decrease in RNA levels of these genes in EhCaBP1-AS cells when grown in presence of tetracycline. These genes are likely to be downstream targets in the EhCaBP1-mediated signaling pathway. Some of the false positive genes, that is genes identified by mRNA display but failed to show differential expression in reverse northern analysis, were also sequenced. Of the two genes analyzed, one was found to be aldehyde dehydrogenase (ADH).
Fig. 18.

A. Differentially expressed cDNAs were identified, excised and reamplified using same primer set as used for first round of display after band excision as described in Materials and Methods. The PCR products were separated on a 1% agarose gel.

B. Reverse northern analysis of the reamplified bands. Equal amounts of DNA obtained by amplification of excised bands as described in A, were spotted on two strips of nylon membrane and hybridized separately. RNA was isolated from EhCaBP1-AS cells after 48 h with or without tetracycline treatment. cDNAs were prepared from the RNA with RT and $[^{32}P]$dATP. Equal amount of radioactivity was used for each hybridization with the membrane containing amplified and displayed cDNA. Arrows show the bands selected for cloning in pGEM Teasy vector for sequencing and northern analysis. Star indicates EhCaBP1 cDNA.

C. Expression of identified cDNAs by Northern analysis. RNA was isolated from indicated cells grown in presence of 20 μg/ml hygromycin with or without 5 μg/ml tetracycline (added at 0 h) for 48 h. For each analysis 20 μg of total RNA was used and separated on a 1% agarose gel at 3V/cm for 3 h by glyoxal/DMSO method as described in Methods. Hybridization was carried out with $[^{32}P]$ labeled inserts of the cDNAs cloned in pGem Teasy. Band size is indicated on left.
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<th>Homologue/Domain</th>
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</table>
3.6. Subcellular localization of EhCaBP1

3.6.1. Biochemical fractionation

Subcellular localization and intracellular distribution of EhCaBP1 was studied using different approaches. The cytoplasmic and membrane fractions were first separated by ultracentrifugation and then western blot analysis was carried out to localize EhCaBP. The result is shown in Fig. 19. EhCaBP was essentially found in the cytoplasmic fraction with negligible amount in the pelleted membrane fraction in most of the cell-lines. However, substantial amount of the protein was also detected in the pelleted membrane fraction in over-expressing EhCaBP1-S cells. This was not due to carry over material as the pellet was washed twice before solubilizing in the PAGE-loading buffer. This suggested that EhCaBP1 may be associated with some component of the membrane which is detectable only when it is overexpressed.

3.6.2. Immunolocalization

Immunostaining was originally carried out using a polyclonal antibody against recombinant EhCaBP1. It has been recently observed that this antibody also recognizes EhCaBP2, an isoform discovered recently. Some of the experiments have been repeated with EhCaBP1-specific antibody and essentially similar results have been obtained. Confocal microscopy of immunostained cells clearly showed the presence of EhCaBP not only in cytoplasm and vacuoles but also in pseudopods and membrane ruffles. In overexpressing EhCaBP1-S trophozoites, high level of EhCaBP1 could be clearly visualized whereas the level was much lower in antisense blocked EhCaBP1-AS cells (Fig. 20). The leading edges of the cells can also be labeled with actin binding phalloidin and an antibody against myosin Ib (Voigt H et al., 1999). At the leading edges EhCaBP was found to co-localize with these proteins suggesting that EhCaBP1 is also present in areas where actin and atypical myosin 1 are present. (Fig. 21).

3.6.3. Expression of actin, myosin Ib and PAK in EhCaBP1-S and EhCaBP1-AS cells

Expression of different proteins, such as actin, myosin Ib and PAK (p21 activated serine/threonine kinase) known to be involved in endocytosis, was determined by western blot analysis using specific antibodies. The amount of actin and PAK were found to be about the same in both EhCaBP1-S and EhCaBP1-AS cells in presence or absence of tetracycline based on densitometric analysis of three separate western blots (Fig. 22A and C). Similar analysis revealed
Fig. 19. Subcellular localization and immunodetection of EhCaBP in transfected trophozoites. *E. histolytica* transfected cells (10⁶) grown for 48 h in presence of 20 μg/ml hygromycin with or without 5 μg/ml tetracycline were used for making lysate and subcellular fractionation as described in Materials and Methods. The pellet fraction after ultracentrifugation contained mainly membrane fraction whereas the cytoplasmic materials are in the supernatant fraction. These proteins were separated on a 12% SDS-PAGE followed by electrophoretic transfer and immunostaining with specific antibody. Lane ‘s’: cytoplasmic fraction and lane ‘p’: membrane fraction. Polyclonal anti-EhCaBP antibody recognizes a 14 kDa (indicated by arrow to the left of each panel) band in cytoplasmic fraction of trophozoites.
Fig. 20. Comparative expression of EhCaBP in transfected trophozoites. Transfected cells grown for 48 h in presence of 20 µg/ml hygromycin with or without 5 µg/ml tetracycline, were transferred to prewarmed, acetone-washed coverslips for 10 min at 37°C. Fixing was done with 3.7% paraformaldehyde/PBS followed by permeabilization with 0.1% Triton/PBS and staining as described in Materials and Methods. Anti-EhCaBP antibody at 1:50 dilution was used. Cells were subjected to similar conditions and fixed optical settings (63X objective, 98 µm pinhole, 1 µm stacks at 488 nm) for confocal microscopy.
Fig. 21. Distribution of EhCaBP, actin and myosin Ib in *E. histolytica* trophozoites. Cells grown for 48 h, were transferred to pre-warmed, acetone-washed cover-slips for 10 min at 37°C. Paraformaldehyde/PBS fixed cells were permeabilized with 0.1% Triton/PBS followed by double-staining with indicated anti-EhCaBP and myosin Ib antibodies or rhodamine-phalloidin as described in Material and Methods. Secondary antibodies used for staining were alexa-green conjugated anti-rabbit antibody for EhCaBP and CY3 conjugated anti-rabbit antibody for myosin Ib. Bar: 5μm. Panel A: double-stained for EhCaBP and actin, B: double stained for Myosin Ib and EhCaBP. DIC is the differential interference contrast image. Arrow indicates the enriched colocalization of EhCaBP with actin/myosin Ib on pseudopod.
Fig. 22. Actin, myosin Ib and PAK levels in transfected cells. Transfected cells were maintained in 20 μg/ml hygromycin with or without 5 μg/ml of tetracycline for 48 h as described in Materials and Methods. Total cellular lysate (50 μg) was separated on 10% SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane. For immunodetection either a monoclonal antibody against actin or a polyclonal antibody against *E. histolytica* myosin Ib and PAK were used. Antibody detection was with anti-mouse IgG or anti-rabbit IgG coupled to horseradish peroxidase (HRPO). The bound proteins were detected using the ECL system.
two fold increase in myosin Ib levels on tetracycline induction of both sense and antisense cells (Fig. 22B). This suggests that EhCaBP1 may not be directly involved in regulation of expression of these genes.

3.6.4. Colocalization of Actin with EhCaBP in phagocytic cups

Immunofluorescence studies showed that EhCaBP1 is distributed evenly all over the cytoplasm and around vacuoles in non-phagocytosing cells. In order to establish the role of EhCaBP1 in the formation of phagocytic cups, relative localization of actin and EhCaBP1 during the different stages of phagocytosis of RBC was observed in different cell lines. On incubation with RBC, the cells showed various stages of engulfment (Fig. 23A). During the engulfment and formation of primary endocytic vesicles, actin was initially found surrounding the crown like structure, termed the phagocytic cup (red). Though EhCaBP1 was found all over the cytoplasm as mentioned before, immunostaining revealed substantial level of this molecule around the phagocytic cup (green). Merging of actin and EhCaBP1 stains showed yellow color surrounding the phagocytic cup suggesting that actin and EhCaBP1 co-localize during the formation of endocytic cup. After engulfment of RBC and formation of the phagocytic vesicles, EhCaBP1 was found to gradually disperse into cytoplasm away from phagosomes. The ability to form phagocytic cups was significantly inhibited in antisense-blocked cells. There was also no significant accumulation of actin in the cortical region below plasma membrane suggesting that these cells are not capable of formation of phagocytic cups, a process needed to initiate endocytosis (Fig. 23D).

3.6.5. Inhibition of actin dynamics

Jasplaklinolide affects actin dynamics by inhibiting depolymerization leading to the formation of G-actin. EhCaBP1 and actin were stained in cells treated with jasplaklinololide at 10 μg/ml for 30 min (Fig. 24A). These cells also showed co-localisation of both EhCaBP and actin. Jasplaklinolide induced the formation of brightly staining aggregates of actin and the retention of cytoplasmic extensions. The cells were also treated with cytochalasin D, an inhibitor of actin dynamics. This causes barbed-end of the actin filaments to cap. Thus bright patches of short actin filaments are seen on staining. Cytochalasin D treatment of E. histolytica cells abolished co-localization pattern for EhCaBP1 with actin (Fig. 24B). After treatment, F-actin showed a coarse punctuated pattern consisting of dense arrays of F-actin. Both these inhibitors did not
Fig. 23. Distribution of EhCaBP and actin during erythrophagocytosis. Cells were grown for 48 h with or without 5 µg/ml of tetracycline as described before and then were transferred to prewarmed, acetone-washed coverslips for 10 min at 37°C before incubation with human red blood cells for 10 min. The cells were then fixed with paraformaldehyde/PBS followed by permeabilization with 0.1% Triton/PBS. Actin and EhCaBP were labeled with phalloidin (red) and alexa-green conjugated second antibody, respectively. Bar: 5µm.
Fig. 24. Distribution of actin and EhCaBP in jasplaklinolide and cytochalasin D treated cells. The cells were grown for 48 h, before incubation at 37°C with jasplaklinolide at 10 μM/ml for 30 min (A), cytochalasin D at 10 μM/ml for 15 min (B) or DMSO for control (C) in a microfuge tube in a reaction volume of 1 ml. The cells were pelleted and fixed before immunostaining with rhodamine-phalloidin (red) and anti-EhCaBP antibody (in a reaction volume of 500 μl) as described in Materials and Methods. Finally cell-pellet was diluted in DABCO for laying on glass-slide. Secondary antibody used for staining EhCaBP was alexa-green conjugated anti-rabbit IgG. Bar: 5μm
Results

affect the distribution of EhCaBP1 in trophozoites. The data suggests that co-localization may involve interaction of EhCaBP1 with F-actin.

3.6.6. EhCaBP1 co-localization with PAK

p21s, active in their GTP-bound forms, mediate growth-factor-induced morphological changes involving actin-based cellular structures. The Pak family of serine/threonine kinases are known to be activated by binding to the GTP-bound form of Cdc42 or Rac1, which are small GTPases of the Rho family that are involved in regulating the organization of the actin cytoskeleton. To investigate whether EhCaBP1 participates in a Rac signaling pathway leading to actin organization, we examined the localization of EhCaBP and PAK in *E. histolytica* cells. Both EhCaBP and PAK were found in the pseudopods and merging of the two stains revealed co-localization (Fig. 25). It appears from the data that EhCaBP is present along with many other proteins that are involved in pseudopod and endocytic cup formation.

3.7. Interaction of EhCaBP1 with F-actin

The confocal microscopic analysis indicated that EhCaBP1 and actin may be interacting with each other which may have a role in the formation of endocytic cups. The EhCaBP1 in presence of calcium forms a compact structure, which in turn binds to a number of proteins by its linker domain, like calmodulin and many other calcium-binding proteins. It is likely that actin may be one of EhCaBP1-binding proteins. In order to show this binding in *in vitro* a number of studies were carried out.

3.7.1. Immunoprecipitation with anti-EhCaBP antibody

The complex containing binding proteins can be immunoprecipitated along with EhCaBP1 using anti-EhCaBP antibody. The immunoprecipitated material was separated on a SDS-PAGE gel and then analyzed by western blot immunostaining as described in Materials and Methods. Antibodies against a number of proteins thought to be involved in endocytosis were used to identify proteins co-immunoprecipitated with EhCaBP1. Only actin was visualized in the western blot (Fig. 26A). No immunostained band was observed when antibodies against myosin II, myosin IB, or profilin were used suggesting that EhCaBP1 binds actin and is present in a complex (Fig. 26B).
Fig. 25. Distribution of EhCaBP and PAK in *E. histolytica* trophozoites. Cells were grown for 48 h with or without 5 μg/ml of tetracycline as described before and then were transferred to prewarmed, acetone-washed coverslips for 10 min at 37°C. The cells were then fixed with paraformaldehyde/PBS, permeabilized with 0.1% Triton/PBS followed by immunostaining with anti-PAK and anti-EhCaBP antibody. PAK and EhCaBP were labeled with CY3 conjugated anti-rabbit second antibody and alexa-green conjugated second antibody, respectively. Bar: 5μm. Arrow indicates the enriched colocalization of EhCaBP and PAK on pseudopod.
Figure 26. Binding of EhCaBP1 to actin in in vitro.

A. Coimmunoprecipitation of amebic proteins with anti-EhCaBP antibody. E. histolytica cell lysate was prepared from $10^6$ cells and was used for immunoprecipitation using anti-EhCaBP antibody and protein A beads, as described in Materials and Methods. The immunoprecipitates were eluted from beads by boiling the beads in 1X SDS-PAGE buffer and separated on a 10% SDS-PAGE gel followed by electrophoretic transfer and immunostaining with anti-actin antibody. The bound protein was detected using the ECL system. Lanes are: 1, immunoprecipitated amebic proteins; 2, control precipitation with just protein A beads; 3, total lysate (10 μg protein). Molecular weight is indicated to the left.

B. Coimmunoprecipitation and western analysis as in (A) with anti-myosinII, anti-myosinIlb and anti-profilin antibody. Lanes are: 1, immunoprecipitated amebic proteins; 2, control precipitation with just protein A beads; 3, total lysate (10 μg protein).

C. Cosedimentation of EhCaBP1 with F-actin. Purified recombinant Eh CaBP1 (5 μM), α-actinin (5 μM) or bovine serum albumin (5 μM) were incubated with F-actin (5 μM), in sedimentation buffer, followed by ultracentrifugation to separate the soluble and pellet fractions as described in Materials and Methods. Pellet (p) and supernatant (s) were resolved in 15% SDS-PAGE followed by coomassie blue staining. Arrow indicates the position of soluble EhCaBP1.
3.7.2. Co-sedimentation of EhCaBP1 and actin

In order to confirm binding of EhCaBP1 with F-actin a co-sedimentation experiment was carried out. Actin filaments were incubated with purified EhCaBP1 and the filaments were pelleted by ultracentrifugation followed by fractionation by SDS-PAGE and staining. Under the same condition a known actin binding protein α-actinin, was found to co-sediment with actin as it was observed in the pellet fraction (Fig. 26C, lane 8). In absence of actin, EhCaBP1 was not observed in the pellet fraction (Fig. 26C, lane 4). However, upon incubation with actin, EhCaBP1 was readily detected in the pellet fraction (Fig. 26C, lane 6). In contrast, BSA could not be pelleted down with actin (Fig. 26C, lanes 1 and 2). This showed a direct interaction between actin and EhCaBP1 as has been observed in the case of actin and α-actinin.

3.7.3. Solid-phase assay for determination of $K_d$ of actin-CaBP interaction

It is likely that EhCaBP1 interacts with both F- and G-actin. In order to characterize the binding to G-actin a solid-phase assay was developed. In this assay actin was coated onto plastic wells of a microtitre plate and EhCaBP1 was incubated at concentrations ranging from 1.7 μM to 10 μM. The amount of EhCaBP1 bound was determined by an enzyme-based immunodetection procedure. As shown in Figure 27A, EhCaBP1 binding to actin was found to be saturable with maximal binding occurring at 5 μM. The association was determined as a function of bound/free amount of EhCaBP1. The dissociation constant ($K_d$) was calculated as 2.6 +/- 0.3 μM from the slope of the scatchard plot (Fig. 27B).

3.7.4. Binding of Actin to EhCaBP2 and mutant EhCaBP1

The specificity of the binding of EhCaBP1 was tested by studying binding of EhCaBP2 to actin using both co-sedimentation (Fig. 28A) and solid phase binding assays. EhCaBP2 is an isoform of EhCaBP1 with 85% identity at the amino acid level. The major difference between the two proteins is in the central linker region. The results of both the assays showed that there was no significant binding of EhCaBP2 with actin. Since the central linker region has diverged maximally between the two proteins the role of the central linker region in actin binding was further checked by using a central linker deleted EhCaBP1 ($ΔcenEhCaBP1$). The mutant EhCaBP1 also did not get co-sedimentated with actin and failed to bind actin in the solid phase.
Fig. 27. Kinetics of binding of EhCaBP1 to actin.
A. Saturation curve for EhCaBP1-actin interaction. Different wells of a microtiter plate were coated with 50 µl of 5 µM actin overnight at 4°C. After blocking with BSA, EhCaBP1 was added at indicated concentrations ranging from 10 µM to 0.1 µM, followed by incubation with anti-EhCaBP antibody. The amount of bound EhCaBP1 was determined anti-rabbit Alkaline Phosphatase (AP) linked IgG. The amount of product formed was detected at 405 nm.
B. Scatchard plot of the data in A.
A. Cosedimentation of EhCaBP1, central-linker deleted EhCaBP1 (ΔcenEhCaBP1) and EhCaBP2 with actin. Purified EhCaBP1, ΔcenEhCaBP1 or EhCaBP2 (5 μM), were incubated with F-actin (5 μM) in sedimentation buffer, followed by ultracentrifugation to separate the soluble and pellet fractions as described in Materials and Methods. Pellet (p) and supernatant (s) were resolved in 15% SDS-PAGE followed by Coomassie blue staining. Arrow indicates the position of soluble EhCaBP1 (14 kDa).

B. Solid phase assay for EhCaBP1, ΔcenEhCaBP1 and EhCaBP2 binding to actin. Briefly, different wells of a microtiter plate were coated with 50 μl of 5 μM actin overnight at 4°C. After blocking with BSA, EhCaBP1 was added at indicated concentrations. The amount of bound EhCaBP1 was detected with anti-rabbit Alkaline Phosphatase (AP) linked IgG. The amount of product formed was detected at 405 nm.

C. Calcium requirement for binding of EhCaBP1 to actin. Binding was carried out by solid phase assay in presence of excess calcium (5 mM) or EGTA (2 mM). The plot shows the relative mean intensity signal obtained from three independent experiments. EhCaBP1 bound in absence of externally added calcium/EGTA was taken as 100% value.

Fig. 28. Binding of a mutant EhCaBP1 and EhCaBP2 to actin.
Results

assay (Fig. 28B). The results suggest that EhCaBP1 specifically binds actin and that the interaction is through the central linker region.

The role of calcium ions in this binding was investigated by carrying out the solid phase binding reaction in presence of EGTA. There was 40-50% reduction in the amount of EhCaBP1 bound in presence of EGTA (Fig. 28C) suggesting that calcium ions play an important role as expected.

3.7.5. Atomic force microscopy of the F-actin.

F-actin is a long protein polymer that plays a crucial role in most forms of biological movement. All muscle tissue and most intracellular cytoskeletal systems are based on arrays or networks of actin filaments. Actin interacts with a very wide variety of other proteins, including molecular motors (myosins) and various anchoring, sequestering, and cross-linking molecules. We used AFM to investigate the interaction of EhCaBP1 with actin.

Reproducible images were obtained under several different conditions. Two representative images of actin filaments are shown in Fig. 29A, B. F-actin was seen to have varied lengths and branching patterns. The line plot on the actin strand gives the height and thickness. Actin has an average height of 2 nm and width of 35-45 nm. With EhCaBP1, the F-actin consistently had lesser strand thickness (20-30 nm) and height (0.8-1 nm). Two representative images of F-actin in presence of EhCaBP1 are shown in Fig. 29C, D.

3.7.6. Immunogold AFM of actin-EhCaBP1

To demonstrate EhCaBP1 binding to actin, anti-EhCaBP1 antibody and 10 nm gold-conjugated secondary antibody was used. Immunogold was found localized all over the strand as well as on unpolymerized actin, thus giving a decorated pattern of actin measuring 300-350 nm in diameter and 30 nm height in AFM as shown in Fig. 30A. F-actin or EhCaBP1 alone, failed to give any pattern (Fig. 30B). AFM images of the immunolabeled actin strands further demonstrate and corroborates the result obtained by solid-phase assay and co-sedimentation assay that EhCaBP1 binds to actin.
Figure 29. AFM micrographs of F-actin. 5μM actin was polymerized in polymerization buffer in presence or absence of EhCaBP1 as described in Materials and Methods. The sample was diluted 1:5000 times and mounted on freshly cleaved mica for microscopy. Panels are:
A. 2 dimensional (2D) view, 2 μm scan of actin filament
B. 3 dimensional (3D) top view, 0.8 μm scan view of actin filament
C. 2D view, 2 μm scan of actin filament polymerized in presence of EhCaBP1
D. 3D top view, 0.8 μm scan of actin filament polymerized in presence of EhCaBP1
Figure 30. Immunogold labeling of actin. Actin (5 μM) was polymerized in presence of EhCaBP1 (5 μM), followed by laying on freshly cleaved mica at 1:5000 dilution. Sample was fixed with paraformaldehyde prior to immunostaining with anti-EhCaBP1 antibody and gold conjugated secondary antibody as described in Materials and Methods. Panel A: actin filament polymerized in presence of EhCaBP1; B: actin alone. Scan size: 10 μm.
3.8. Pathogenesis of *E. histolytica*: role of EhCaBP1

**Interaction of Caco2 epithelial cell monolayer with *E. histolytica* trophozoites**

One of the important steps that leads to symptomatic infection is the attachment of *E. histolytica* trophozoites to host intestinal epithelial cells. The human intestinal cell line Caco2 has been used as a model for studying amoeba-cell interaction *in vitro* (Li E *et al.*, 1994). Different transfected cell lines of *E. histolytica* were used to decipher the role of EhCaBP1 in pathogenesis by determining their ability to interact with Caco2 cells.

**3.8.1. Adhesion**

A monolayer of Caco2 cells on a coverslip was incubated with $3 \times 10^5$ *E. histolytica* cells at $37^\circ$C in a CO$_2$ incubator. After 15 min the medium was removed for estimation of non-adherent amoebae. The number of adherent trophozoites, expressed as percent adhesion, was taken as the difference between the initial number of amoebae added ($3 \times 10^5$) and the total number of amoebae in the removed medium and in the wash liquid. Each experiment was carried out in triplicate on two different days. No significant difference in percent adhesion was observed for both EhCaBP1-S and EhCaBP1-AS cells in presence or absence of tetracycline, suggesting that EhCaBP1 may not play any role in adhesion (Fig. 31).

**3.8.2. Cytolytic properties**

The cytolytic property of the cells was investigated by using the fluorescent dye 2', 7'-bis (carboxyethyl)-5(6)-carboxyfluorescein acetomethyl ester (BCECF-AM). *E. histolytica* cells ($10^5$) were incubated with a Caco2 cell monolayer prelabeled with BCECF-AM for 1 h. After the incubation period, the release of the fluorescent dye from dead target cells was measured in a fluorescence microtiter plate reader using excitation and emission wavelengths of 485 and 538 nm, respectively. The results showed that there is no significant difference in the amount of dye released with EhCaBP1-S and EhCaBP1-AS cells in presence or absence of tetracycline (Fig. 32). Calcium chelator EGTA, reduced the ability of amoebae to kill the epithelial cells which may be due to absence of influx in target cells, a prerequisite for target cell death.

Immunolabeling was also used to visualize the extent of killing of Caco2 cells by transfected amebic cells. Phalloidin red and Cd6 antibody were used for labeling epithelial and amebic cells respectively. Confocal analysis of fixed cells showed different stages of destruction of the target
Fig. 31. Adhesion of transformed *E. histolytica* trophozoites to Caco-2 cells. Washed *E. histolytica* cells ($10^5$) were incubated Caco2 monolayer ($10^6$ cells) for 15 min. Total cell count of trophozoites in the supernatant were used to calculate the value of bound amoebae.
Fig. 32. Cytolytic activity of transformed amoebae. Indicated *E. histolytica* cells (10⁵ cells in 1 ml) washed with DMEM medium were incubated with Caco2 monolayer (10⁶ cells) prelabeled with 2',7'-bis (carboxyl)-5(6)-carboxyfluoresceinacetoxymethyl ester (BCECF-AM), as described in Materials and Methods. After the incubation period, the release of the fluorescent dye was measured in a microtiter plate reader using excitation and emission wavelengths of 485 nm and 538 nm, respectively.
Results

cells by amoebae irrespective of the amebic cell-type (Fig 33). Our findings showed that overexpression or antisense blocking of EhCaBPI did not affect the cytolytic ability of *E. histolytica*.
Fig. 33. Destruction of Caco2 cells by transfected amoeba. Indicated *E. histolytica* cells (10⁵) grown with or without tetracycline, washed with DMEM medium were incubated with Caco2 monolayer (10⁶ cells) grown on glass cover-slip, for 15 min, followed by fixing and immunostaining with CD6 antibody (for amoeba) and rhodamine-phalloidin (for Caco2) as described in Materials and Methods. Micrograph showing Caco2 monolayer destruction by antisense blocked trophozoites grown with or without tetracycline on 15 min interaction. Amoeba was stained with CD6 antibody (red) and Caco2 with rhodamine-phalloidin. Confocal sections are at 1µm height.